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(54) Title: HIGH LEVEL AMPLIFICATION AND EXPRESSION OF EXOGENOUS DNA

(57) Abstract

A method for producing high level expression of a selected protein and cell line and vectores useful therein. This method involves incorporating an exogenous ADA gene and an exogenous gene coding for a desired protein into a cell line containing an endogenous ADA gene.

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HIGH LEVEL AMPLIFICATION AND EXPRESSION OF EXOGENOUS DNA

Background

This invention relates to a method and unique expression vectors that use heterologous adenosine deaminase (ADA) DNA as a selectable marker for transformation and/or as a co-amplifier of DNA coding for an exogenous protein in a host cell containing endogenous ADA.

Transformation is a commonly-employed genetic engineering procedure in which new genetic material is acquired by eukaryotic or procaryotic cells by the incorporation of exogenous DNA sequences coding for a desired protein, polypeptide, and the like. Ordinarily, the number of cells in a population undergoing transformation which actually incorporate the exogenous DNA is quite low.

These problems can be obviated by transforming the cell with a selection marker in addition to the exogenous DNA sequence. Depending upon whether and how closely the selection marker is linked to the exogenous protein-encoding DNA, cells carrying the selection marker will also contain the exogenous DNA. Using appropriate conditions, cells transformed with the selection marker can be distinguished from cells that have not incorporated the exogenous DNA. Selection involves the use of DNA encoding an easily-identifiable marker, for example, resistance to an antibiotic. Upon transformation, the cell population is examined for the presence of the marker. Those cells which have successfully incorporated the marker DNA will exhibit the marker identity (e.g. survival in media containing the antibiotic) and those cells which have failed to incorporate

the marker will not exhibit the marker feature (e.g. will die upon exposure to the antibiotic).

The level of exogenous protein expressed by transformed cells can be substantially increased wh... DNA encoding an amplifiable gene as well as a select... marker is included in the transformation process. Ampl... cation of a gene involves exposing the transformed ce... environmental pressure sufficient to require the ce... produce more copies of the amplifiable gene for sur... Accordingly, the use of gene amplification for the ... level expression of exogenous genes is an important techn...

The marker/amplification system most extensively employs the gene for dihydrofolate reductase (DHFR), a ... ubiquitous gene found in many cell lines. Exposing ... transformed with DHFR-encoding DNA to cytotoxic co... trations of methotrexate (MTX) encourages the ce... amplify DHFR to survive. Cells which survive th... selection procedure have many copies of the DNA en... DHFR. When the DHFR gene is on a plasmid containing ... sequence for another gene, that gene generally ... amplified as well. Thus when transforming a cell w... vector containing a DHFR gene and an exogenous gen... DHFR behaves as a selectable marker to enable the ide... cation of those cells which have incorporated the ... from those cells which have not and also is capa... being itself amplified and consequently amplifyin... exogenous DNA. The use of the DHFR gene both as a selec... le and amplifiable marker has become widespread for de... transformed cell lines.

However, in practice, the DHFR system has demonst... general utility only with one cell line, a Chinese ha... ovary line which is deficient in DHFR (CHO DHFR⁻). [Ur... et al, Proc. Natl. Acad. Sci. U.S.A., 77:4216-4220 (19...)] Cell lines containing endogenous DHFR genes cannot be emplo... because the endogenous DHFR prevents selection of those cell

containing the DHFR and exogenous gene-containing vector.

A mutant DHFR gene has been reported which purportedly can be expressed when inserted into cell lines containing endogenous DHFR. [Simonson, C.C. et al., Proc. Natl. Acad. Sci. U.S.A., 80: 2495-99, (1983)]. However, these cell lines cannot be significantly amplified and are of marginal utility in attempting to obtain the high level of exogenous polypeptide desired from transformed cells. The construction [illegible] a selectable marker enabling the use of DHFR in cell lines possessing the DHFR gene has been reported by Murray, M.J. et al., Mol. Cell. Biol. 3: 32-43 (1983). However, obtaining the optimal conditions necessary for expression of exogenous proteins in such cell lines has proven difficult.

Thus, expression and amplification of exogenous protein with the DHFR system has been limited to a single cell line, which is not always the cell line of choice for producing the desired protein. Other cell lines produce specific proteins at a greater level than, or will grow better than, CHO DHFR$^-$ under specified conditions. Other systems for amplifying and expressing heterologous DNA in a variety of different cell lines remain an unfulfilled need in the art.

Summary of the Invention

As one aspect of the present invention, it is surprisingly discovered that an exogenous adenosine deaminase [illegible]DA) gene may be used as a selectable and amplifiable marker in cell lines containing an endogenous ADA gene. A gene encoding ADA is present in virtually all mammalian tissues, but is not an essential enzyme for cell growth. [See Shipman, C. Jr., et al., Science 200: 1163-1165 (1978); Hirschorn, R. et al., Proc. Natl. Acad. Sci. U.S.A. 73: 213-217 (1976)]. The method of the present invention thus makes possible the amplification of exogenous DNA coding for a desired protein in a wide variety of ADA+ eucaryotic cells, particularly mammalian cells. This method involves

incorporating an exogenous ADA gene and a heterologous [illegible] coding for a desired protein into a cell line containing endogenous ADA gene. Cells containing the exogenous [illegible] gene and the heterologous protein gene are then sele[illegible] and the genes amplified. Finally, the heterologous pro[illegible] gene is expressed and the desired protein recovered.

As another aspect of the present invention, a [illegible] line is provided for use in the ADA amplification meth[illegible] The cell line is produced by transforming a cell contai[illegible] endogenous ADA with an exogenous gene coding for ADA and [illegible] exogenous gene coding for the desired protein and coamplify[illegible] these exogenous genes. The resulting cell line with [illegible] amplified ADA and protein genes may then be cultured accor[illegible] to the present invention. High levels of the desi[illegible] protein are expressed thereby. The ADA gene so emplo[illegible] can be the presently known sequence, of either human [illegible] or murine ADA. Depending on the use to which the prot[illegible] is to be put, however, other species ADA genes may be u[illegible] in analogous fashion.

As a further aspect of the present invention, no[illegible] vectors are provided which incorporate exogenous ADA ge[illegible] and exogenous genes coding for a desired protein. The[illegible] vectors contain polyoma or retroviral sequences and can [illegible] employed to transform ADA$^+$ cells or cell lines for use [illegible] the method of the invention to produce the desired prote[illegible]

Unlike the DHFR amplification system which requi[illegible] use of a DHFR$^-$ cell line, the ADA amplification meth[illegible] makes possible the employment of many ADA+ cells and ADA$^+$ c[illegible] lines that will grow best under specific conditions and [illegible] preferentially express a desired product, as well as AD[illegible] cells and ADA$^-$ cell lines. Use of cell lines that wi[illegible] process the protein more effectively or properly (e.g., [illegible] making-post translational modifications such as gammacarbox ylation) is also possible.

Brief Description of the Drawings

Figure 1 illustrates the structure of plasmid p9ADA5-29.

Figure 2 illustrates the structure of plasmid pFVXM.

Detailed Description of the Invention

According to the method of the present invention, a cell line containing an endogenous ADA gene is transformed with a foreign ADA cDNA. The production of ADA cDNA would follow a procedure analogous to that for cloning any other gene. [See generally Maniatis, T. et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1982); Toole, J. J. et al., Nature 312: 342-47 (1984)]. The sequences of human ADA cDNA and mouse derived ADA cDNA have been determined [See Wiginton, D. A. et al., Nucl. Acids Res. 12: 1015-1024 (1984); Valerio, D. et al., Gene 31: 147-153 (1984); Yeung, C. et al., J. Biol. Chem., 258: 15179-15185 (1983)]. ADA cDNA can be placed into a mammalian expression vector using techniques well known by those having ordinary skill in the art.

The cell to be transformed may be any ADA+ eucaryotic cell, including yeast protoplasts and various bacterial cells, but is preferably a nonfungal cell and most preferably, is a stable mammalian cell line. Useful in the practice of this invention are HeLa cells, melanoma cell lines such as the Bowes cell line, mouse L cells, mouse fibroblasts, mouse NIH 3T3 cells, and the like. Cell lines that are known to stably integrate ADA and other genes into their chromosomal DNA are also desirable, e.g., Chinese hamster ovary (CHO) cell lines, human hepatoma Hep G2 cell lines and mouse myeloma cell lines, depending upon the other requirements placed upon the cell line.

Exogenous genes are normally not expressed as well as endogenous chromosomal genes. It is thus a surprising

aspect of the invention that it is possible to transfo cells with exogenous ADA and select for transformants c terized by significantly higher levels of ADA expr in comparison to endogenous ADA+ cells which under gene amplification as a result of the same selection p ures. ADA is unique because in most cells it is exp at a very low level. Introduction of an efficient exp ADA gene renders those transformed cells capable of tion. However, a few ADA+ cell lines express high levels than produced in most cell lines, e.g., those from gastrointestinal and thymus tissues, and sh avoided. [See Lee, P.A., Dev. Biol. 31: 227-233 (Barton, R. et. al., Cell Immunol. 49: 208-214 (198 Y. et. al., Thymus 4: 147-154 (1982)].

The population of cells exposed to transfe conditions is then processed to identify the transfor i.e., the small subpopulation which exhibit the phe of the ADA selection gene. The cells in the cultu screened for the phenotype by placing selection press the cell. The specific selection method to be used determined by the person of ordinary skill in th Specific known methods for selecting for increa expression are summarized below. The skilled arti adapt these and other known methods to select for containing exogenous ADA.

One such ADA selection method involves the adenosine analogues. Cells can be selected for resi to cytotoxic adenosine analogues 9- -D-arabinofur adenine (Ara-A) or 9- -D-xylofuranosyl adenine (Multiple step selection in either Ara-A or Xyl-A resul cell populations with increased ADA activity. [See, C. et. al., J. Biol.Chem. 258: 8330-8337 (1983)]. the ability to catalyze the irreversible conversi these adenine analogues to their respective inosine de tives which are eventually detoxified by removal of

ribose by purine nucleoside phosphorylase to yield hypoxanthine. Because cells may become resistant to these analogues by loss of adenosine kinase activity, not all surviving cells will have increased levels of ADA. [V. L. Chan et. al., Somatic Cell Genet. 7: 147-160 (1981); Yeung, et. al. supra]. However, the frequency of loss of adenosine kinase is usually low in cells which contain a diploid complement of the adenosine kinase gene.

A selection protocol which selects for the presence of adenosine kinase [Chan, T. et. al., Somatic Cell Genetics 4: 1-12 (1978)] has been modified so that it can also be used to select for increased expression of ADA. [See Yeung, C. et. al., supra 15179-15185 (1983)]. In contrast to the first procedure, all surviving cells exhibit increased levels of ALA. Adenosine kinase is selected for in the presence of AAU (adenosine, alanosine, uridine). Under this growth condition, cells are blocked in de novo AMP (adenosine monophosphate) biosynthesis by alanosine and require adenosine kinase to convert adenosine to AMP. Since adenosine depletes phosphoribosylpyrophosphate (PRPP) which results in the inhibition of endogenous pyrimidine synthesis, the medium is supplemented with uridine. [See Green, H. et. al., Science 182: 836-837 (1973); Ishii, K., et. al., Cell Sci 13: 429-439 (1973)]. However, when the adenosine concentration is increased 11-fold (hereinafter 11-AAU selection) the high concentrations of adenosine become cytotoxic and ADA is required to alleviate the toxicity. [See Fox, I.H. et. al., Ann Rev Biochem 47: 655-686 (1978)].

Once functional ADA is required for cell growth, (R)-deoxycoformycin (dCF), an antibiotic demonstrated to be a tight binding transition-state analogue inhibitor of ADA (kd=2.5 x 10^{-12}), can be used to select for amplification of the ADA gene. [See Agarwal, R. P. et. al., Biochem. Pharmacol. 26: 359-367 (1977); Frieden, C. et. al., Biochem.

19: 5303-5309 (1980)]. For the cell to survive in t[illegible] systems, ADA is required in higher levels than most c[illegible] produce. Growth of cells in 11-AAU in the presence [illegible] sequentially increasing concentrations of dCF, [illegible] cells which contain a high degree of ADA expression a[illegible] result of amplification of the ADA gene. [See Yeu[illegible] C., supra at 8338-8345 (1983)].

Yet another selection method employs deoxyadeno[illegible] as a carbon source. Cells can also be made growth depen[illegible] on ADA activity by blocking purine de novo synthesis [illegible] azaserine and feeding cells 2-deoxyadenosine as a pu[illegible] source. [See Fernandez-Mejia, et. al., J. Cell Phy[illegible] 120: 321-328 (1984)]. Deoxyadenosine is available a[illegible] general purine source only if converted to deoxyinosine [illegible] ADA. As a result, cells can be selected for increa[illegible] activity by growth in azaserine with increasing concentrati[illegible] of dCF. The medium is supplemented with deoxycyti[illegible] [See Thelander, L. et. al., Ann. Rev. Biochem. 48: 1[illegible] (1979)].

A similar approach has been described by Hunt, [illegible] et al., J. Biol. Chem. 258: 13185-13192 (1983), uti[illegible] adenosine as the sole carbon source. Under these condi[illegible] dCF resistant variants of Novikoff rate hepatom[illegible] which require functional ADA, were isolated by [illegible] adenosine kinase-deficient cells in a medium cont[illegible] adenosine as the sole carbon source with stepwise incr[illegible] concentrations of dCF. This procedure yields cells [illegible] have amplified the ADA gene 320-fold. [See also, H[illegible] P.A. et al., Somatic Cell Genet. 8: 13185-1392 (1983)[illegible]

In any given population a certain number of [illegible] containing an endogenous ADA gene will express a [illegible] level of ADA than other cells. Thus, the degree of sel[illegible] pressure will effect the sensitivity of distingui[illegible] cells transformed with exogenous ADA from cells contai[illegible] higher levels of ADA expression from an endogenou[illegible]

gene. Accordingly, it is desired that one select for those cells expressing ADA at a five-fold increase and more preferably a ten-fold increase over that typically found expressed by cells containing endogenous ADA genes.

Transformants exhibiting higher levels of ADA than endogenous ADA+ cells can be obtained by using vectors that result in more efficient expression of the heterologous gene. Cells can be transformed by use of a vector that contains both the ADA gene and the product gene as well as one or more other elements such as enhancers, promoters, introns, accessory DNA, a polyadenylation site and three prime non-coding regions. [See Clark, S.C. et al., Proc. Natl. Acad. Sci. USA 81: 2541-2547 (1984); see also Kaufman, R. J., Proc. Natl. Acad. Sci. USA 82: 689-693 (1985)]. These may be obtained from natural sources or synthesized by known procedures. Basically, if the components found in DNA are available in large quantity, e.g., components such as viral functions, or if they are to be synthesized, e.g., polyadenylation sites, large quantities of vectors may be obtained with appropriate use of restriction enzymes by simply culturing the source organism, digesting its DNA with an appropriate endonuclease, separating the DNA fragments and identifying the DNA containing the element of interest and recovering the same.

Various vector systems including polyoma or retrovirus systems can be used provided they express the ADA produced by the exogenous ADA gene at a level above that expressed by cells containing endogenous ADA. Preferably 5-times greater expression is desired, more preferably 10-times.

Two classes of vectors can be employed in transformation herein. Transformation with unlinked vectors, that is, one vector containing the exogenous ADA gene and another vector containing the desired exogenous product gene, can be accomplished simultaneously. Methods for facilitating cellular uptake of DNA are well known to those

skilled in the art. Considerably better transf... efficiencies result from transformation with a mola... of product gene to ADA gene, preferably on the ... 10:1 or higher.

To most effectively obtain coamplification of ... product gene, the use of linked vectors in which ... and product genes are covalently bound is prefer... coding strands of the ADA and product genes are ... joined by directly ligating the product stop codon ... to the ADA gene start codon. The genes may be ... through an oligodeoxyribonucleotide bridge. The ... should be free of termination or start codons, ... palindromes to reduce the probability of forming RNA ... loops. Alternatively, one may transform with a ... vectors containing a plurality of discrete product...

The vectors for use in producing the cells or ... useful in the method of the present invention are ... supercoiled, double-stranded circular constructs, ... in which vectors are obtained from the standard prok... cloning procedure. However, the vectors may be lin... i.e., covalently cleaved at one point, incidental ... steps such as ligation to genomic accessory DNA.

One preferred vector is plasmid p91023(B) whi... deposited with the American Type Culture Collection, ... Parklawn Drive, Rockville, MD in E. coli MC1061 und... deposit number 39754. The deposited vector can be m... by using EcoR1 digestion to delete the CSF gene and re... it with an ADA gene. p91023(B) has been used fo... expression of ADA in CHO cells and Baby Hamster ... cells, BHK.

As one embodiment of the invention, a vector cont... a polyoma origin of replication and transcription en... in operative association with an exogenous ADA gen... exogenous gene coding for a desired protein, is prov... For example, the p90123 vector can be modified using t...

niques known in the art to delete the SV40 enhancer element and replace it with the polyoma, ADA and protein coding sequences. The resulting plasmid can respond to polyoma virus early T antigen and replicate in the presence of polyoma T antigen in mouse cells. This vector can then be introduced into a polyoma transformed mouse cell line which is expressing high levels of T antigen.

The polyoma system is analogous to that used in the COS system while having significant advantages thereover. COS cells are SV40 transformed monkey kidney cells, which express T antigen from SV40. Upon introduction of a plasmid that contains an origin of replication for SV40 into COS cells, the T antigen will act on that SV40 origin of replication and will replicate very high copy numbers of the plasmid. Because the plasmid replicates to such a high copy number (about 50,000 copies per cell), the cells die rapidly and they can only be cultured for up to two weeks.

Polyoma replicates about an order of magnitude less efficiently than the COS system thereby providing better conditions for cell survival. Mouse cells in which polyoma can replicate, can be selected to express T antigen from polyoma. A plasmid which encodes for ADA and also has an origin of replication for the polyoma, can be introduced into the mouse polyoma transformed cells. Replication can occur as a plasmid rather than by integration and can range from 1,000 copies to 10,000 copies per cell. As a result of using a polyoma cell line and amplifying it using dCF in the presence of either high levels of adenosine or in the presence of Xyl-A, one should typically obtain a 100-fold higher resistance to dCF than is usually obtained in CHO or BHK.

In another embodiment of the present invention, a novel vector is provided which operatively links retrovirus sequences with an exogenous ADA gene. Group antigen, polymerase and envelope genes are deleted from the retrovirus

and replaced with an ADA gene with the proper tran and packaging signals to direct the envelopment of A into the virus. Such retrovirus construction te are known to those skilled in the art. This virus be transmitted from one cell to another cell. The p of this ADA virus can be screened for by selecting presence of increased ADA expression in other cell vector is particularly desirable because it provi capacity to get the ADA gene into cells with ve efficiency. The copy number may be amplifiabl the initial infection because of the presence of gene. Such retroviral vectors may be used to infe in vivo for use in mammalian gene therapy, as well create the cell lines useful in the present method.

Once the host cell or cell line is transformed vector containing exogenous ADA DNA and an exogeno coding for a desired protein and desired transforma selected, they are screened for ligation of the gene into their chromosomes or for expression of the itself. The product genes which can be used are ess unlimited. Genes for proteins or enzymes having act that are found in the cells of higher animals mammals or vertebrae are the genes of most present herein. Even genes for proteins that may adversely the whole cell by synthesizing toxins or hydrolyzi protein may be employed with procedural modific such as providing antitoxins in the culture medium selecting lower expression levels than would otherw optimum.

Screening for ligation of the product gene accomplished using Southern blot analysis. Screeni expression of the product can utilize standard immun cal, biological or enzymatic assays. Once the transf have been identified, expression of the product gene amplified by subculturing in the presence of a sel

agent in constant or increasing amounts as described above. Presently, the use of the 11-AAU procedure with increasing concentrations of dCF is preferred. Generally this entails (a) selecting one or more cells from the transformant cell population that express the product in a preferential fashion when compared to other cells in the population, (b) culturing the selected cell or cells to a subsequent cell population under conditions designed to select for a change in the expression of the phenotype, and (c) further selecting one or more cells from the subsequent cell population that express the product in a preferential fashion when compared to other cells in the subsequent population. Step (b) advantageously is conducted with a plurality of the step (a) clones.

Although any of the procedures discussed supra can be utilized in both selection and amplification of the transformants, in more preferred embodiments, a combination of different procedures should be utilized. The Xyl-A procedure appears to be both more sensitive and more consistent than the 11-AAU system in selecting for uptake of exogenous DNA. Amplification of the transformants is preferably performed using the 11-AAU selection procedure.

Although the transformants can be grown in any medium, certain precautions are required depending upon the particular procedure utilized as described below. For example, fetal calf serum has much higher levels of endogenous ADA than horse serum. In Xyl-A selection, 3nM dCF is used in the presence of 4.0uM Xyl-A in contrast to 11-AAU selection where 0.01uM dCF is used with 0.03uM dCF in the presence of 1 mM adenosine. Thus when using a selection procedure that only requires very low levels of cytotoxic agent, e.g., Xyl-A, a growth media containing high levels of endogenous ADA, such as fetal calf serum, can detoxify the cytotoxic agent. If the use of fetal calf serum was desired, one could switch selection protocols to a different system, for

example 11-AAU, which uses significantly more of a ... agent and would be minimally effected by fetal ... One could also utilize a separate selection marker.

Alternatively, if one desires to use the Xyl-A ... method, a number of strategies can be used to over... problem. Horse serum could be used instead of fet... serum because it does not contain high levels of en... ADA. However, if use of fetal calf serum is desire... concentrations of Xyl-A can be utilized to mini... effect of the fetal calf serum ADA. Further, one ... the Xyl-A right before selection and continue addi... periodically to replace the Xyl-A detoxified by ... ADA.

The following examples illustrate the u... method of the present invention.

EXAMPLE 1

Construction of p9ADA5-29 and Expression of ADA ... monkey kidney COS cells

The ADA cDNA sequence for expression may be ... from the published human and murine sequences ... above. For example, mouse ADA cDNA, pADA5-29 [See ... al., supra at 15179-15185] was placed into a ... expression vector p90123, which is derived from p... by deleting the CSF gene with EcoRI digestion. ... nucleotide open reading frame in pADA5-29 was exc... NcoI and EcoRI digestion. The ends were filled in ... Klenow fragment of DNA polymerase 1 and blunt-end ... into the EcoRI site of vector p91023. The resultant ... p9ADA5-29 (see Figure 1), contains (from left to righ... adenovirus VA gene (VA), the SV40 origin of repli... including the 72 bp enhancer, the adenovirus virus... late promoter including the adenovirus tripartite lea... and a 5' splice site (AdMLP), a 3' splice acceptor (3'ss), the ADA insert (ADA), the dihydrofolate redu...

insert (DHFR), the SV40 early polyadenylation site (SV40) and the pBR322 sequences needed for propagation in E. coli.

Vector p9ADA5-29, was used to transfect COS-1 cells using the DEAE-dextran procedure. [Kaufman, R. J., Proc. Natl. Acad. Sci. USA, supra]. The transfected cells underwent zymogram analysis which indicated that the cells produced authentic mouse ADA at high levels.

EXAMPLE 2

Selection and Amplification of Cells Transformed with ADA cDNA

DHFR deficient CHO cells, CHO DHFR$^-$, (DUKXB11), were grown in an alpha media with 10ug/ml of thymidine, deoxyadenosine and adenosine. Cells were transfected with pADA5-29 ($25ug/10^6$ cells) as described by Kaufman, R. J., et al., J. Mol. Biol. 150:601-621 (1982). Forty-eight hours post-transfection, cells were plated ($8x10^4$ cells/10cm plate) into either (1) alpha media supplemented with 10ug/ml thymidine, 15ug/ml hypoxanthine, 4uM Xyl-A, with varying concentrations of dCF (2) alpha media supplemented with 10ug/ml thymidine, 10ug/ml deoxyadenosine, 1mM uridine, 1.0mM adenosine and varying concentrations of dCF. Four plates at each dCF concentration level were prepared for both media. The two media used correspond to the Xyl-A selection procedure and a modified 11-AAU selection procedure, 11-AU, respectively. The 11-AAU procedure was altered because CHO DHFR$^-$ cells cannot produce purines de novo, resulting in no need to use alanosine. To avoid detoxification of the cytological agents by the low levels of ADA endogenous to fetal calf serum, 10% fetal calf serum is added just prior to use of the media.

This transfection procedure was also repeated exactly as described above with no exogenous ADA DNA placed into the CHO cell lines to produce mock-transfected CHO DHFR$^-$ cells for comparison. Results of the selection procedures showed that the Xyl-A selection media is more sensitive in indicating

uptake of exogenous DNA than the 11-AU procedure. for DNA uptake is preferably measured using about and about 0.003-0.01uM dCF.

Transformants were amplified using the 11-AAU in combination with increasing levels of dCF as in Yeung, C. et al., supra at 8338-8345, and as above by excluding alanosine. Transformants were in DMEM supplemented with 10% fetal calf serum Island Biological Company) and incubated at transformed CHO DHFR$^-$ cells were grown in the 11-A described above.

Six transformed colonies which were sele by 11-AU selection at dCF concentrations of 0.01 were placed in the above described media. These then exposed to 0.1uM or 0.5uM of dCF respectively. cells not producing large amounts of ADA were killed growth resumed for surviving cells, the cells were several times at the same level of dCF. Then concentration was increased. Cells were exposed step-wise at levels of 0.03uM, 0.1uM, 0.5uM, 1uM, 20uM.

Cells to be analyzed were removed from drug for 1 week and fed with fresh DMEM plus 10% serum before harvest. Cells were harvested by trypsin washed three items with Hank's balanced salt (without Mg^{2+} and Ca^{2+}), and resuspended in twice packed volume of homogenizing medium (10 mM Tris 7.5, 1mM beta -mercaptoethanol, and 1 mM EDTA). The pended pellet was frozen at -20°C, thawed and hom using a motorized Teflon homogenizer. The sample centrifuged twice at 15,000 x g for 30 min to remove The supernatants (containing -1mg of protein/ml applied directly to starch gels. Electrophoresis conducted at 4°C using 200V for 16 hours or 400V hours. Following electrophoresis, the starch

sliced into replica sheets of ~1 mm thickness and histochemically stained for adenosine deaminase activity as described in Sicilano, M. J., et al., Chromatographic and Electrophoretic Techniques (Smith, I., ed.) 4th Ed., vol 2, pp. 185-209 Wm. Heinemann Medical Books Ltd., London (1976); and Harris, H. et al., Handbook of Enzyme Electrophoresis in Human Genetics, North/Howland, Oxford (1976).

This treatment resulted in an amplification for the transformants selected at 0.1uM dCF of about 10-times and for the cells selected at 0.03uM dCF of about 50-times. Further amplification is obtained by continuing to apply selection pressure on surviving cells with step-wise increments of dCF as described above.

EXAMPLE 3

Transformation and Coamplification of ADA with a Product Gene

Plasmid p9ADA5-29, described in Example 1, is mixed with a p91023 (B) derivative, p91023-p, containing a DNA sequence coding for the desired product polypeptide instead of the CSF gene. 50 ug p91023-p is mixed with 0.5 ug p9ADA5-29 and precipitated by the addition of NaOAc (pH 4.5) to 0.3 M and 2.5 vols. of ethanol. Precipitated DNA is allowed to air dry, then resuspended in 2X HEBSS (.5ml) [Chu et al., Gene 13: 197-202 (1981)] and mixed vigorously with .25 M $CaCl_2$ (.5ml) as described in Kaufman, R. J. et al., J. Mol. Biol. supra. The calcium-phosphate-DNA precipitate is allowed to sit 30 minutes at room temperature, and applied to CHO DUKX-B1 cells [Chasin, et al., Proc. Natl. Acad. Sci. USA 77: 4216-4220 (1981)]. The growth and maintenance of these cells has been described in Kaufman et al., J. Mol. Biol. supra and Chasin et al., supra.

The DUKX-B1 cells are subcultured at 5 x 10^5/10cm dish for 24 hours prior to transfection. The media is removed, and the DNA - calcium phosphate precipitate is added to the monolayer. After 30 minutes incubation at room temperature,

5ml of alpha-media (Flow) with 10% fetal calf ... applied and the cells are incubated at 37°C for 4... The media is then removed from the monolayer of ... of alpha-media (Flow) containing 10% glycerol is ... 3 minutes at room temperature (24°C) and then rem... the cells are rinsed and fed with alpha-media co... 10% fetal calf serum, 10 ug/ml each of thymidine, ad... deoxyadenosine, penicillin and streptomycin. ... later the cells are subcultured 1:15 in the selecti... as described above.

Colonies will appear 10-12 days after subcu... into selective media. Two schemes for selection and ...cation can be followed. In the first scheme single i...dent cloned transformants are isolated on the b... uptake of the exogenous ADA DNA and subsequent... clone is propagated under conditions to increase ex... of the product gene i.e., growth in increasing concent... of dCF. In the second scheme pools of multiple ind... transformants are isolated on the basis of uptake... exogenous ADA DNA and are propagated under conditi... increase expression of the product gene, i.e., gro... increasing concentrations of dCF. Then individual... are isolated from the mass selected population and a... for expression of the product gene. Those clones exh... highest levels of product gene expression are grown under conditions to further increase product exp... (i.e., growth in increasing concentrations of dCF ... culture media).

An alternative method of transfecting and coampl... ADA or a product gene is to employ only a p9102... containing both the ADA gene and the product gene in... of the unlinked vectors p91023-p and p9ADA5-9 in th... cedures of this example.

EXAMPLE 4

Selection for Heterologous ADA Genes in Mouse Fibroblast Cells

A plasmid, pXC-ADA, containing the polyoma virus origin of replication and transcriptional enhancer in place of the SV40 origin and transcriptional enhancer in pADA5-29 was derived by the following procedures. Starting plasmid .84.A2.X containing the polyoma regulatory region ligated with an XhoI linker at the Bcl 1 site [See Veldman et al., Mol. Cell Biol. 5:649-658 (1985)] was digested with the restriction endonuclease Bgl 1. The end was rendered flush by a fill-in reaction using T4 DNA polymerase 1 in the presence of 100 uM each dATP, dTTP, dCTP, and dGTP [Maniatis et al. supra]. EcoRI linkers (Collaborative Res.) were applied and the DNA digested with an excess of EcoR1 and XhoI. The resultant DNA was electrophoresed on a 6% polyacrylamide gel using Tris-Borate as a buffer system and the fragment migrating at 370 bases was isolated by electro-elution (Id.).

The 370 bp fragment was ligated to vector pAdD26SVpA#1, described in Kaufman, R. J. et al. Mol. Cel. Biol., supra which was previously digested with XhoI and EcoR1. The resultant plasmid was used to liberate an approximately 400 bp fragment by XhoI and Cla 1 digestion. This fragment, containing 24 bp from pBR322 between the EcoR1 site to the Cla 1 site, was isolated and ligated to pADA5-29 which had been previously digested with XhoI and Cla 1. The DNA was used to transform E. coli HB 101 for tetracycline resistance and colonies were screened by filter hybridization [Grunstein et al. Proc. Natl. Acad. Sci., 72: 3961 (1975)] to a probe prepared by nick translation of the original XhoI-Bgl 1 fragment from p.84.A2.X. Positively hybridizing clones were analyzed and plasmid pXC-Ada was prepared by banding DNA twice in cesium chloride. The structure of plasmid

pXC-Ada was confirmed by analysis after digesti multiple restriction enzymes.

pXC-Ada was transfected into mouse fibroblasts p ously transformed with an origin defective polyom early region (MOP, provided by Claudio Basilico, N.Y. versity School of Medicine) as described by Kaufman, J. Mol. Biol., supra except the cells were propag DME media with 10% fetal calf serum.

The early region of polyoma virus expresses three formation antigens (large, middle, and small T anti which elicit the transformed phenotype. Large T ant elicits replication of plasmids introduced into the fibroblasts containing a polyoma origin of replic [Tyndall et al., Nuc. Acids Res., 9:6231-6250 (1981)]. Forty-eight hours after transfection, cells were subcul at $2X10^5$ cells/dish in media containing 4uM Xyl-A increasing concentrations of dCF. Five plates a concentration were prepared.

After two weeks, both cells transfected with p and mock transfected (no exogenous DNA) had colonies selection in 0.01uM dCF. In 0.03uM dCF, 43 colonies app in the transfected compared to 3 in the mock. This nu decreased for transfected cells to 34 at 0.1uM dCF 15 at 0.3uM dCF. In 0.3uM dCF, 43 colonies appeared in transfected compared to 3 in the mock. This number decr for transfected cells to 34 at 0.1uM dCF and to 15 at dCF. Virtually no colonies were found at these hi levels in the mock cells. Growth of cells at these concentrations of dCF indicates that the transfected have many copies of the plasmid pXC-ADA even without ampl cation by sequential selection in higher concentrat of dCF. Use of pXC-ADA to select for high levels of expression in polyoma transformed in fibroblasts

likely resulted from high plasmid replication driven by the polyoma replication signals.

EXAMPLE 5

Selection for Expression of Retrovirus Transmitting Functional ADA

The retroviral vector pEVX [Kriegler et al., Cell, 38: 483-491 (1984)] was derived from sequences of both Moloney leukemia virus and Harvey Sarcoma virus. pEVX was modified by deletion of the Harvey Sarcoma virus packaging site while still retaining the packaging signal sequences of Moloney leukemia virus which are fully functional [Proc. Natl. Acad. Sci. 72:3961 (1975)].

The resulting plasmid pFVXM [Fig. 2] contains the viral long terminal repeats (LTRs), and an internal polylinker for insertion of heterologous genes. It does not contain the retroviral group antigen (gag), polymerase (pol), and envelope (env) genes. The Bgl II site in this plasmid is unique and is ideal for the insertion and subsequent expression of virions capable of producing the protein encoded by the inserted squence.

Exogenous ADA was prepared for insert into pFVXM, by digesting pADA5-29 with EcoRI and SacI, treating with T4 DNA polymerase to flush the ends, and applying Bgl II linkers (Collaborative Res.). After Bgl II digestion and agarose gel electrophoresis, on approximately 1.8 kb band was isolated. This fragment was ligated to pFVXM, which had previously been digested with Bgl II. Colonies were screened by colony hybridization (Grunstein et al. supra.) to a nick-translated DNA fragment (the original EcoRI and SacI fragment isolated from pADA5-29). DNA was prepared from positively hybridizing clones by restriction endonuclease analysis. One clone, pRetro ADA-1-1, was found to contain the ADA insert in the proper orientation with respect to

the retroviral long terminal repeat (LTR) used [illegible] scription initiation.

pRetro ADA 1-1 DNA was prepared by propag[illegible] E. coli HB101 and DNA banded twice in cesium chlori[illegible] DNA was transfected into mouse fibroblast ψ2 cells [illegible] al., Cell 33: 153-159 (1983)] which contain a d[illegible] Moloney viral genome that cannot be packaged int[illegible] virions. However, the gag, pol, and env polyp[illegible] (which are required for virus production and are [illegible] from pRetro ADA 1-1) are expressed from the d[illegible] genome. Those proteins are sufficient to comple[illegible] functions missing in pRetro ADA 1-1. 48 hours aft[illegible] mediated DNA transfecton of $2x10^6$ ψ2 cells with pRetro ADA 1-1, the cells were subcultured into [illegible] with 0.01M dCF. Three colonies appeared from th[illegible] receiving DNA where no colonies appeared when the [illegible] omitted. One colony, ψ2-ADA, was chosen and anal[illegible] ADA retrovirus production.

The conditioned media from 10^6 cells ([illegible] harvested after 24 hours and after filtration (0.2[illegible] applied to 3T3 cells ($2x10^6$) in the presence of [illegible] polybrene for 2 hours. The virus was then removed[illegible] cells were supplied with fresh media. 48 hours [illegible] confluent 3T3 cells were subcultured 1:10 into me[illegible] taining 4uM Xyl-A and 0.01 or 0.03uM dCF. After [illegible] colonies were counted. The uninfected cells had no [illegible] growing in 0.01 or 0.03uM dCF per $2x10^6$ originally i[illegible] cells. Infected cells had approximately 4000 colo[illegible] 0.01uM dCF and 3000 colonies in 0.03uM dCF. These [illegible] indicate that $>10^3$ infectious units were present p[illegible] culture fluid from the transfected ψ2 cells.

This procedure allows the introduction of an ampl[illegible] vector into cells with a potent selection system to [illegible] cells expressing the heterologous ADA. It should be p[illegible]

by using techniques well known in the field to introduce other genes into the retrovirus in order to also place them into cells. The presence of the exogenous ADA gene allows for potential amplification of the inserted viral DNA. In addition the amplification of the retroviral sequences in the ψ2 cells allows for production of higher titre virus stocks which are essential in order to introduce genes into animals and into humans.

What is claimed is:

1. A method for producing high level expre[illegible] a selected exogenous protein comprising culturing which comprises at least one copy of an endogenou[illegible] coding for ADA, amplified copies of an exogenou[illegible] coding for ADA and amplified copies of an exogenou[illegible] coding for said selected protein.

2. The method according to claim 1 further com[illegible] transforming a cell containing an endogenous gene for ADA with an exogenous gene coding for ADA and an ex[illegible] gene coding for said selected protein and coamplifyi[illegible] exogenous ADA gene with said exogenous protein gene.

3. The method according to claim 2, further comp[illegible] transforming said cell with a single expression v[illegible] comprising said exogenous protein gene and exogenous AD[illegible]

4. The method according to claim 3, further comp[illegible] transforming said cell with a single expression [illegible] on which said exogenous protein gene and said exogen[illegible] gene are covalently linked.

5. The method according to claim 2, further comp[illegible] transforming said cell with one expression vector comp[illegible] said exogenous ADA gene and second expression vecto[illegible] prising said exogenous protein gene.

6. The method according to claim 1, wherein cell is selected from the group consisting of yeast bacterial cell and mammalian cell lines.

7. The method according to claim 6 wherein said mammalian cell lines are selected from the group consisting of Bowes cell line, mouse L cells, mouse fibroblasts, mouse NIH 3T3 cells, human hepatoma Hep G2 cell lines and CHO cell lines.

8. A cell line for use in producing high levels of expression of a selected exogenous protein produced by transforming a cell line which contains an endogenous gene coding for ADA with an exogenous gene coding for ADA and an exogenous gene coding for said protein and co-amplifying said exogenous ADA and protein genes.

9. The cell line according to claim 8, wherein said exogenous gene coding for ADA is selected from the group consisting of murine ADA, human ADA, bacterial ADA and yeast ADA.

10. A vector comprising an exogenous gene coding for ADA in operative association with retrovirus transcription and packaging sequences capable of directing the envelopment of said gene.

11. The vector according to claim 10, further comprising a gene encoding a desired exogenous gene.

12. A vector comprising an exogenous gene coding for ADA and a gene coding for a desired protein in operative association with an adenovirus VA gene, an SV40 origin of replication, an adenovirus major late promoter and an SV40 early polyadenylation site.

13. A vector comprising an exogenous gene cod... ADA, and a gene coding for a desired protein in op... association with a polyoma virus origin of replicat... polyoma virus transcriptional enhancer.

1/2

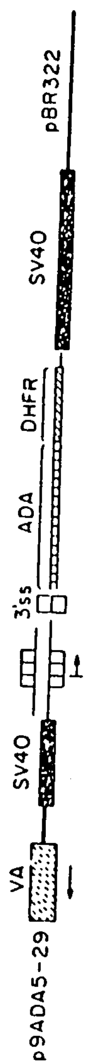

FIG. 1

SUBSTITUTE SHEET

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pFVXM

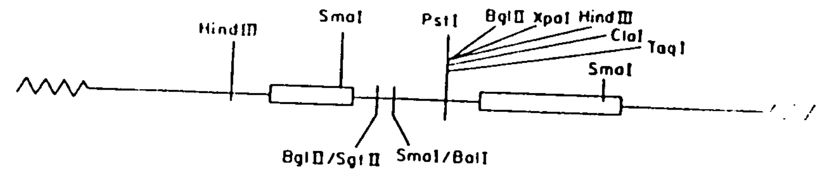

FIG. 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00934

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4 C12P 21/00, C12N 5/00, C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/68, 172.3, 227, 240, 317 935/32, 34, 57, 70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Chemical Abstracts Data Base (CAS) 1967-1986; Biosis Data Base 1969-1986, Lexpat, 1975-1986. Keywords: adenosine deaminase, c[illegible], cloning, plasmid, vector, gene, gene amplification,

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 4,399,216 (AXEL ET AL.) 16 August 1983, see column 3, lines 43-61 column 6, lines 1-5 column 8, lines 36-55	1-9
Y	Proceedings of the National Academy of Sciences, U.S.A., Volume 80, issued December 1983 (Washington, D.C. U.S.A.) Wiginton et al., "Cloning of cDNA sequences of human adenosine deaminase" pages 7481-7485	1-13
Y	Journal of Biological Chemistry, Volume 258, Issued December 25, 1983 (Bethesda, Maryland, U.S.A.) "Amplification and molecular cloning of murine adenosine deaminase gene sequences" pages 15179-15189, see especially pages 15179 and 15183	1-13
X Y	Proceedings of the National Academy of Sciences, U.S.A., Volume 82, issued February 1985, (Washington, D.C. U.S.A.) Friedman, "Expression of human adenosine deaminase EC 3.5.4.4 using a transmissible murine retrovirus vector system" pages 703-707	10 1-9,11

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 July 1986	22 JUL 1986
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Karen Maurey Karen Maurey

0013

International Application No PCT/US86/00934

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proceedings of the National Academy of Sciences, U.S.A., Volume 81, issued April 1984, (Washington, D.C. U.S.A.) Clark, et al. "Human T-cell growth factor: partial amino acid sequence, cDNA cloning and organization and expression in normal and leukemic cells" pages 2543-2547, see especially page 2545	12-13
A	Proceedings of the National Academy of Sciences, U.S.A., Volume 82, issued February 1985 (Washington, D.C., U.S.A) Kaufman "Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors" pages 689-693 see especially page 690	12-13